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Exploiting Cascade Reactions in Bienzyme Layer-by-Layer Films

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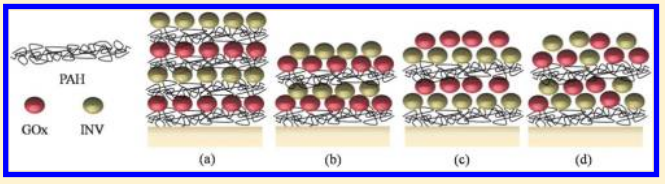
Supporting Information

ABSTRACT: Control of supramolecular architectures inherent in layer-by-layer (LbL) films allows for synergistic effects between the film components, as in reactions under controlled conditions. In this paper we show that a bienzyme system can be produced in LbL films, in which the product of a reaction catalyzed by invertase (INV) is used in a catalysis process involving glucose oxidase (GOx). As a proof of principle, simultaneous detection of glucose and sucrose is demonstrated for an optimized film architecture comprising poly allyllamine hydrochloride (PAH) alternated with INV + GOx layers $(PAH/(GOx + INV))_{10}$. Though the sensitivity for sucrose is low because the product from the reaction with invertase has low electroactivity, the results confirm that cascade reactions can be performed in a controlled manner in LbL films with possible applications in sensors and bioelectrochemical devices.



1. INTRODUCTION

Immobilization of biomolecules, including proteins and enzymes, in layer-by-layer (LbL)[1,2] films has been exploited for many years[3−5] in various applications, such as biosensing.[6−9] There are various reasons why the LbL technique has been widely used for biomolecules, but perhaps the main ones are possible control of the molecular architecture and mild conditions for film fabrication that are amenable to preserve the bioactivity.[10] LbL films are obtained with the alternate adsorption of charged species from aqueous solutions on a solid substrate, and therefore, electrostatic interactions are normally the driving force for spontaneous adsorption.[11] The method was originally conceived to exploit these electrostatic forces in polyelectrolytes[12] but has been extended to a variety of materials (see refs 13 and 14 for reviews) and may also include other driving forces for adsorption, such as H bonding[15,16] or electrochemical coupling.[17] Examples of control in film architecture are abundant and for numerous applications. For example, polymers in LbL films have been applied in light-emitting diodes (LEDs),[18] graphene[19] and carbon capsules[20] were used for sensing, and enzymes have been combined with metallic nanoparticles[18,21] or nanotubes[18,22−24] to enhance the sensitivity of biosensors.

With regard to immobilization of enzymes, the LbL method is just one of several possible approaches. Others include covalent binding, entrapment in gels and membranes or polymer matrices, and cross-linking.[25,26] The physical adsorption in LbL films is advantageous for flexibility in the choice of materials to constitute the films, though stability may be lower than in the chemical methods. Upon immobilization, enzymes are often stabilized, being thus less sensitive to denaturing agents.[25,26] One main advantage of immobilization—in comparison to systems with the enzyme in solution, free to move in a reaction medium—is the facilitated recovery and reuse in continuous processes, in biosensors, bioreactors, and other industrial applications.[25,26]

The concept of exploiting multiple enzymes in a single LbL film was proposed by Lvov et al.[3] but has apparently received little attention for only a few papers have touched on this point.[9,23,27−29] In this paper we revisit the concept with a systematic search for film architectures containing glucose oxidase (GOx) and invertase (INV). The main goal is to demonstrate that the product of the reaction with one enzyme can be used as a substrate for the other enzyme. Film growth for the various architectures was monitored with UV−vis absorption and fluorescence spectroscopy, while the enzyme activity was inferred from chronoamperometry measurements.

2. MATERIALS AND METHODS

Glucose oxidase (GOx) from *Aspergillius niger* (E.C. 1.1.3.4), 118 000 units/g, isoelectric point at pH 4.2, pK_a 8.2,[30,31] and optimal activity at pH 3.5−6.5,[32] invertase (INV) grade VII, from Baker's Yeast (E.C. 3.2.1.26), 401 000 units/g, isoelectric point at pH 4.5, pK_a 6.8,[33] and optimal activity at pH 4.0−8.0,[34] and PAH were purchased from Fluka, Sigma, and Aldrich, respectively. Glucose (Synth) and sucrose (Synth) were used without further purification, and glucose solutions were allowed to mutarotate overnight. All other reagents were of analytical grade and used without further purification.

The LbL films were assembled onto quartz and ITO-coated glass (indium−tin oxide, one-side coated on glass by Delta Technologies), previously modified with a PB film potentiostatically

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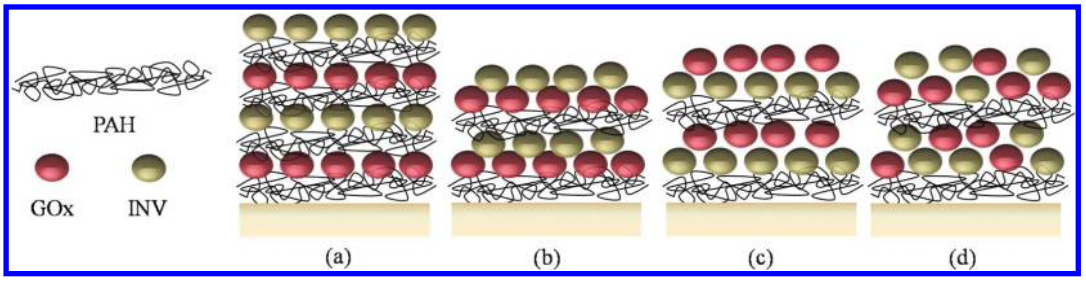

Figure 1. LbL architectures (a) PAH/GOx/PAH/INV, (b) PAH/GOx/INV, (c) PAH/INV/GOx, and (d) PAH/GOx + INV.

deposited at a potential of +0.40 V during 400 s from aqueous 2×10^{-3} mol L^{-1} $K_3(Fe(CN)_6)$ + 2×10^{-3} mol L^{-1} $FeCl_3$ solutions in 0.1 mol L^{-1} KCl + 0.01 mol L^{-1} HCl. After deposition, the modified electrodes were rinsed with Milli-Q water (pH 5.9) and immersed into a solution containing 0.1 mol L^{-1} KCl + 0.01 mol L^{-1} HCl, where the electrode potential was cycled between 0.0 and 1.0 V at a scan rate of 0.05 V s^{-1} versus saturated calomel electrode (SCE) until a stable voltammetric response was obtained.[7]

Both PAH and INV solutions for LbL film fabrication had a concentration of 0.5 mg mL^{-1}, while a concentration of 10 mg mL^{-1} was found—in a systematic optimization procedure—to be optimum for the GOx solution. On the basis of the film architecture chosen, GOx and INV solutions were prepared in a phosphate buffer (10 mmol L^{-1}, pH 6.3) or acetate buffer (10 mmol L^{-1}, pH 5.3), with the PAH solution having the same pH adjusted by adding NaOH 10 mmol L^{-1}. LbL films were assembled by immersing the quartz (to study film growth) or ITO/PB-modified substrates (for electrochemical measurements) alternately into the polycationic solution (PAH) for 4 min and anionic solutions (GOx) or (INV) for 4 and 20 min, respectively, interspersed by a washing step of 1 min in buffer solution. This time period was sufficient for substantial amounts of PAH, GOx, and INV to be adsorbed. The buildup of the multilayers was monitored at each deposition step with a Genesys 6 UV−vis spectrophotometer. Immobilization was confirmed through fluorescence spectroscopy using a Hitachi FL 4500 spectrophotometer. To minimize effects from the substrate morphology,[35] a 2-bilayer cushion film of PAH/PVS (polyvinyl sodium sulfonate) was grown onto quartz substrates for spectroscopy analysis.

Electrochemical measurements were performed with an Autolab PGSTAT 30 (Ecochemie). All potentials were measured with reference to an SCE electrode, and platinum was employed as the auxiliary electrode. Tests of enzyme activity toward glucose and sucrose were carried out in a conventional three-electrode electrochemical cell (10 mL) containing phosphate or acetate buffer solution (10 mmol L^{-1}) as electrolyte at 0.0 V. Before all batch amperometric experiments, the potential of each modified electrode was held at the operating value, allowing the background current to decay to a steady state value.

3. RESULTS AND DISCUSSIONS

The main purpose of this work is to verify whether multireactions can be performed in a bienzyme LbL film containing GOx and INV. GOx activity can be monitored with the glucose catalysis process depicted below:

$$\beta\text{-glucose} + O_2 \xrightarrow{\text{GOx}} \text{gluconicacid} + H_2O_2 \qquad (1)$$

Since H_2O_2 can be monitored amperometrically but at a high potential, we employed a Prussian Blue modified electrode for detecting H_2O_2 at lower potentials, then avoiding the response from possible interfering compounds, such as ascorbate, acetaminophen, and ureate. As for the INV activity, it can be monitored through sucrose catalysis:

$$\text{sucrose} + H_2O \xrightarrow{\text{INV}} \alpha\text{-glucose} + \beta\text{-frutose} \qquad (2)$$

The latter catalysis process does not produce any electroactive compound, and the two mechanisms can be combined in a final scheme:

$$\begin{aligned} &\text{sucrose} + H_2O \xrightarrow{\text{INV}} \alpha\text{-glucose} + \beta\text{-frutose} \\ &\alpha\text{-glucose} + O_2 \xrightarrow{\text{GOx}} \text{gluconicacid} + H_2O_2 \end{aligned} \qquad (3)$$

Here we focus on the lower activity of GOx toward α-glucose (0.64% compared to its main substrate β-glucose as 100%)[36] instead of using the enzyme mutarotase that interconverts the α and β isomers, a common step in sucrose biosensors. The enzymes GOx and INV were immobilized with PAH in LbL films deposited on ITO covered with Prussian Blue with the following architectures, represented in Figure 1. The number of bilayers was kept at 10, considered suitable for guaranteeing sufficient material for electrochemical detection without creating diffusion barriers that are inevitable in very thick films.

In subsidiary experiments we produced LbL films containing either GOx or INV to check whether the enzymes remained active after immobilization. Growth of PAH/GOx and PAH/INV LbL films is depicted in Figure S1 (Supporting Information). Also, the ability of PAH/GOx and PAH/INV LbL films to detect glucose and sucrose, respectively, is demonstrated in Figures S2 and S3 (Supporting Information). It is important to mention that sucrose could only be detected by adding GOx to the electrolyte solution because—as discussed before—reaction with INV does not generate electroactive species.

As for the architectures mentioned above, growth of 10-tetralayer PAH/GOx/PAH/INV LbL films was monitored by measuring the absorption at 280 nm vs the number of layers, with the results shown in Figure 2. Because the band at 280 nm was very small for the PAH/GOx and PAH/INV LbL films, we confirmed that deposition was indeed successful by taking the fluorescence spectrum of a 10-tetralayer film, as shown in the inset of Figure 2. The increase in absorption with the number of layers can be fitted with the sum of two exponential functions, which indicates that two processes with distinct characteristic times are involved in adsorption of the enzyme. This behavior differs from that of PAH/GOx LbL film and the linear growth for the PAH/INV LbL film (see Supporting Information). It seems

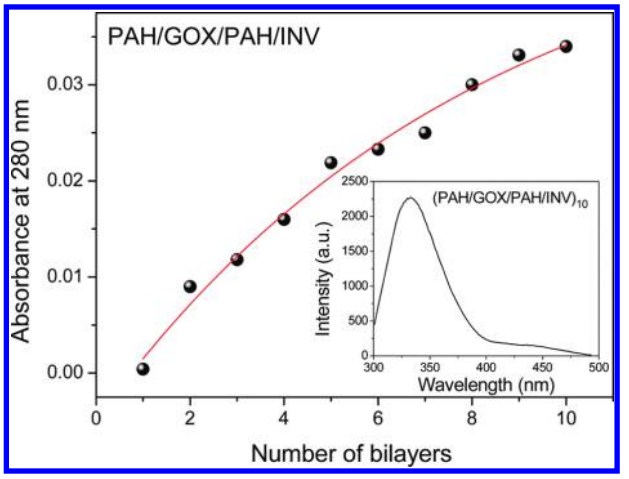

Figure 2. Absorption maximum at 280 nm for PAH/GOx/PAH/INV LbL film versus number of deposited layers. (Inset) Fluorescence spectrum for a 10-tetralayer PAH/GOx/PAH/INV LbL film, obtained with excitation at 280 nm. Emission with a maximum at 333 nm is assigned to the phenylalanine and tryptophan chromophores of the enzymes.

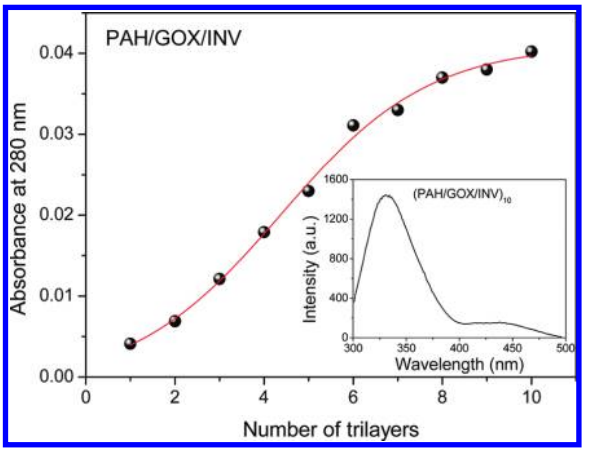

Figure 3. Absorption maximum at 280 nm for PAH/GOx/INV film versus number of deposited trilayers. (Inset) Fluorescence spectrum of a 10-trilayer PAH/GOX/INV LbL film, obtained with excitation at 280 nm.

that this departure is due to the simultaneous presence of both enzymes in the film.

Growth of a 10-trilayer PAH/GOx/INV LbL film is depicted in Figure 3. Again, we confirmed adsorption of the enzymes by measuring the fluorescence spectrum for a 10-trilayer film, shown in the inset. Note that INV layers could be deposited on top of a GOx layer even though at the pH used both enzymes were above their isoelectric points, suggesting that adsorption was governed by secondary interactions such as H bonding. The curve for absorption versus the number of trilayers can be fitted with a sigmoidal Boltzmann function, which is not typical of LbL films. This may have been caused by rearrangement of the biomolecules in the layers after a given number of layers were deposited.

The LbL films with the $(PAH/INV/GOx)_n$ architecture had a maximum n of 3 because only a small number of layers could be deposited. When n was larger than 4, adsorption was followed by desorption and the LbL films could not grow (results not shown). The LbL films with GOx and INV in the same layer

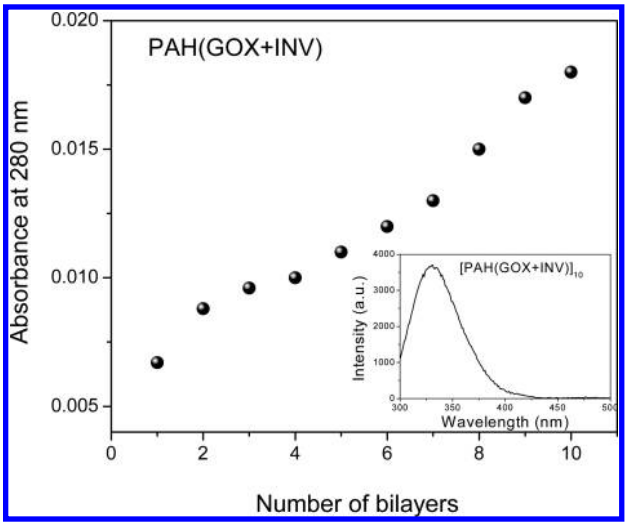

Figure 4. Absorption maximum at 280 nm for PAH/(GOx + INV) LbL film at pH 5.3 versus number of deposited bilayers. (Inset) Fluorescence spectrum for a 10-bilayer PAH/(GOx + INV) LbL film, with excitation at 280 nm.

were deposited from a solution containing a mixture of GOx and INV, whose pH was 5.3. Figure 4 shows another peculiar behavior for growth of the $PAH/(GOx + INV)_{10}$ LbL film at pH 5.3, with a large increase in the first few layers and then again with a larger number of layers. It seems that—similarly to the case of the PAH/GOx/INV LbL films of Figure 3—some rearrangement of the enzyme conformation took place. Adsorption of enzymes was confirmed in the fluorescence spectrum shown in the inset.

Among all the architectures tested, only the one with GOx and INV in the same layers responded to glucose and sucrose simultaneously. Additionally, appreciable electrochemical response was only obtained using INV and GOx concentrations of 1 and 5 mg mL^{-1}, respectively. All measurements were carried out with successive additions of sucrose followed by glucose additions in the electrolyte solution (acetate buffer pH 5.3). Figure 5 shows a large change in the amperometric signal when glucose was added, while the changes caused by sucrose were much smaller. The latter ones, nevertheless, increased with sucrose concentration (as shown in the zoomed figure in the inset). The reason why the effect from sucrose was much smaller is related to the poor activity of immobilized GOx toward the α-glucose generated during sucrose catalysis.

Onda et al.[37] investigated multienzymatic LbL films containing glucose oxidase (GOx) and glucoamylase (GA) to detect starch through a sequential enzymatic reaction. GA hydrolyzed starch to glucose, which was then converted by GOx to gluconic acid and H_2O_2. The film architecture polyelectrolyte/GOx/polyelectrolyte/GA showed high activities, consistent with the order of the sequential enzymatic reactions, while the film containing polyelectrolyte/(GA+GOx) showed the lowest yield. These results were attributed to the fact that GA adsorption from the mixed solution could have been insufficient for sequential enzymatic reactions. In contrast, in our study the film architecture polyelectrolyte/GOx/polyelectrolyte/INV was not efficient for the sequential reaction, while the film containing polyelectrolyte/(GOx+INV) was. This difference in behavior illustrates that the importance of the film architecture and type of enzyme

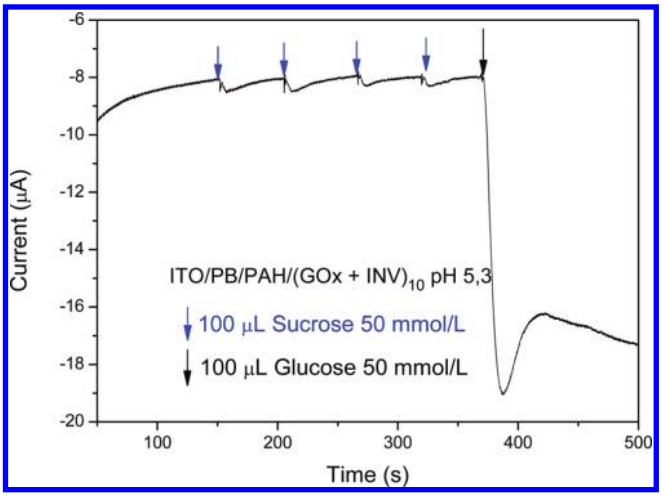

Figure 5. Amperometric response for an ITO/PB/PAH/$(GOx + INV)_{10}$ prepared at pH 5.3 and obtained at 0.0 V in a phosphate buffer at pH 6.3. Addition of glucose and sucrose is indicated.

used for sequential reactions depend on the ease with which reaction products can diffuse through the film.

It is also significant that only one of the architectures could be used for simultaneous detection. It seems that unless GOx is exposed to the electrolyte, the α-glucose resulting from the reaction catalyzed by INV cannot be detected, especially owing to its small electroactivity.

4 . CONCLUSIONS

The versatility of the LbL technique was exploited in this work to produce various film architectures containing the enzymes glucose oxidase (GOx) and invertase (INV). Film growth depended on the order with which the layers of enzymes were combined, and in some cases the amount of material adsorbed increased exponentially or according to a sigmoid Boltzmann function, probably owing to changes in conformation of the enzymes. We showed that in an optimized bienzyme LbL film it is possible for the product of one catalysis process to be used as substrate for the other enzyme. This was proven with chronoamperometry measurements with ITO/PB-modified electrodes that were capable of detecting glucose and sucrose simultaneously. In this optimized architecture GOx had to be exposed to the electrolyte. Most significantly, the results presented here demonstrate that cascade reactions may be induced in multienzyme LbL films with important implications for many applications in biotechnology.

■ ASSOCIATED CONTENT

Supporting Information. Growth and amperometric response of PAH/GOx and PAH/INV LbL films were investigated separately. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ REFERENCES

(1) Ladam, G.; Schaaf, P.; Decher, G.; Voegel, J. C.; Cuisinier, F. J. G. *Biomol. Eng.* **2002**, *19*, 273–280.
(2) Cassier, T.; Lowack, K.; Decher, G. *Supramol. Sci.* **1998**, *5*, 309–315.
(3) Lvov, Y.; Ariga, K.; Ichinose, I.; Kunitake, T. *J. Am. Chem. Soc.* **1995**, *117*, 6117–6123.
(4) Onda, M.; Lvov, Y.; Ariga, K.; Kunitake, T. *Biotechnol. Bioeng.* **1996**, *51*, 163–167.
(5) Ariga, K.; Ji, Q. M.; Hill, J. P. *Adv. Polym. Sci.* **2010**, *229*, 51–87.
(6) Zhao, W.; Xu, J. J.; Chen, H. Y. *Electroanalysis* **2006**, *18*, 1737–1748.
(7) Ferreira, M.; Fiorito, P. A.; Oliveira, O. N.; de Torresi, S. I. C. *Biosens. Bioelectron.* **2004**, *19*, 1611–1615.
(8) Gao, Q.; Guo, Y.; Zhang, W.; Qi, H.; Zhang, C. *Sens. Actuators, B* **2011**, *153*, 219–225.
(9) Yao, H.; Hu, N. *J. Phys. Chem. B* **2010**, *114*, 9926–9933.
(10) Siqueira, J. R., Jr.; Caseli, L.; Crespilho, F. N.; Zucolotto, V.; Oliveira, O. N., Jr. *Biosens. Bioelectron.* **2010**, *25*, 1254–1263.
(11) Decher, G. *Science* **1997**, *277*, 1232–1237.
(12) Decher, G.; Hong, J. D.; Schmitt, J. *Thin Solid Films* **1992**, *210*, 831–835.
(13) Ferreira, M.; Zucolotto, V.; Ferreira, M.; Wohnrath, K.; Oliveira Jr., O. N. Layer-by-layer (LBL) and Langmuir-Blodgett (LB) films from nanoparticles and complexes. In *Encyclopedia of Nanoscience and Nanotechnology*; Nalwa, H. S., Ed.; American Scientific Publishers: Los Angeles, CA, 2003; Vol.4, pp 441−465.
(14) Oliveira Jr., O. N.; Zucolotto, V.; Ferreira, M.; Mattoso, L. H. C.; Riul Jr, A. *Nanostructured films employed in sensors In Supramolecular Engineering of Conducting Materials*; Research Signpost: Kerala/India, 2005, Vol.11, pp 399−425.
(15) Ferreira, M.; Onitsuka, O.; Stockton, W. B.; Rubner, M. F. *Photonic Optoelectronic Polym.* **1997**, *672*, 437–444.
(16) Raposo, M.; Oliveira, O. N. *Braz. J. Phys.* **1998**, *28*, 392–404.
(17) Li, M.; Ishihara, S.; Akada, M.; Liao, M.; Sang, L.; Hill, J. P.; Krishnan, V.; Ma, Y.; Ariga, K. *J. Am. Chem. Soc.* **2011**, *133*, 7348–7351.
(18) Ho, P.; Kim, J.; Burroughes, J.; Becker, H.; Li, S.; Brown, T. *Nature* **2000**, *404*, 481–484.
(19) Ji, Q.; Honma, I.; Paek, S.-M.; Akada, M.; Hill, J. P.; Vinu, A.; Ariga, K. *Angew. Chem., Int. Ed.* **2010**, *49*, 9737–9739.
(20) Ji, Q.; Yoon, S. B.; Hill, J. P.; Vinu, A.; Yu, J.-S.; Ariga, K. J. *Am. Chem. Soc.* **2009**, *131*, 4220–4221.
(21) Yang, M. H.; Yang, Y.; Yang, H. F.; Shen, G. L.; Yu, R. Q. *Biomaterials* **2006**, *27*, 246–255.
(22) Gan, N.; Hou, J.; Hu, F.; Zheng, L.; Ni, M.; Cao, Y. *Molecules* **2010**, *15*, 5053–5065.
(23) Du, Y.; Chen, C.; Li, B.; Zhou, M.; Wang, E.; Dong, S. *Biosens. Bioelectron.* **2010**, *25*, 1902–1907.
(24) Chen, H.; Xi, F.; Gao, X.; Chen, Z.; Lin, X. *Anal. Biochem.* **2010**, *403*, 36–42.
(25) Brady, D.; Jordaan, J. *Biotechnol. Lett.* **2009**, *32*, 1639–1650.
(26) Sheldon, R. A. *Adv. Synth. Catal.* **2007**, *349*, 1289–1307.
(27) Wang, J.; Liu, G.; Lin, Y. *Analyst* **2006**, *131*, 477–483.
(28) Chen, Z.; Xi, F.; Yang, S.; Wu, Q.; Lin, X. *Sens. Actuators, B* **2008**, *130*, 900–907.
(29) Kreft, O.; Prevot, M.; Mohwald, H.; Sukhorukov, G. *Angew. Chem., Int. Ed.* **2007**, *46*, 5605–5608.
(30) Zou, Y.; Xian, C.; Sun, L.; Xu, F. *Electrochim. Acta* **2008**, *53*, 4089–4095.
(31) Voet, J. G.; Coe, J.; Epstein, J.; Matossian, V.; Shipley T. 1981, *20*, 7182-7185.
(32) Bankar, S. B.; Bule, M. V.; Singhal, R. S.; Ananthanarayan, L. *Biotechnol. Adv.* **2009**, *27*, 489–501.

(33) Waheed, A.; Shall, S. *Biochim. and Biophys. Acta* **1971**, *242*, 172–186.
(34) Arica, M. Y.; Bayramoglu, G. *J. Mol. Catal., B: Enzym* **2006**, *38*, 131–138.
(35) Lobo, R. F. M.; Pereira-da-Silva, M. A.; Raposo, M.; Faria, R. M.; Oliveira, O. N. *Nanotechnology* **2003**, *14*, 101–108.
(36) Leskovac, V.; Trivic, S.; Wohlfahrt, G.; Kandrac, J.; Pericin, D. *Int. J. Biochem. Cell Biol.* **2005**, *37*, 731–750.
(37) Onda, M.; Lvov, Y.; Ariga, K.; Kunitake, T. *J. Ferment. Bioeng.* **1996**, *5*, 502–506.